c) two splice acceptor sites between the promoter and the ß-galactosidase gene, and

d) a polyadenylation site of SV40.

REMARKS/ARGUMENTS

I. Status of the Claims

Claims 1-10 are pending in the instant case and stand variously rejected under 35 U.S.C. §112, first and second paragraphs. Applicants respectfully traverse the rejections and request reconsideration of the claims in light of the remarks presented herein below.

II. Formalities and Amendments to Specification

The Examiner presented a number of objections to the format of the specification and drawings. Applicants have addressed each of these objections as described in brief herein below.

The Examiner objected to the drawings as filed indicating that the drawings were not correctly identified by separate labels. Attached herewith as Appendix B is a submission of formal drawings for this case. The drawings are appropriately labeled thereby addressing the objections set out by the Examiner. Furthermore, the heading for the drawings and the drawing descriptions for Figs. 1-5 have been amended as suggested by the Examiner and to correct typographical errors. A marked-up version of the drawing descriptions are provided herewith as Appendix A.

The abstract of the specification was objected to as being presented in more than one paragraph and exceeding the word limit for an abstract. This has been rectified by the above amendment. The text of the former abstract has been added to the specification as a "Summary of the Invention." The various other objections to the specification have been addressed by amendment. See Appendix A. No new matter has been added by any amendment of the above amendments. Applicants request that in light of these amendments, the Examiner's objections to the specification be withdrawn.

Regarding the certified copy of the priority EP 97 106503.2, Applicant is in the

process of obtaining a certified copy and will provide it to the USPTO when available.

Regarding incorporation by reference of EP 97 106503.2, since the specification satisfies the requirements of 35 U.S.C. 112, first paragraph, without the disclosure of EP 97 106503.2 for the pending claims, EP 97 106503.2 cannot be said to contain "essential material" in regards to the pending claims. However, Applicant reserves the right to amend the specification to include the disclosure of EP 97 106503.2 should such disclosure be essential to future pending claims or deemed to be essential to the present claims by the Examiner.

Claims 4 and 5 have been amended to correct a typographical error regarding the spelling of "*typhii*." Such change is supported throughout the specification, including at least at page 26, line 20.

III. Rejections under 35 U.S.C. §112, first paragraph should be withdrawn.

In section 10 of the Office Action (paper 17, page 7), the Examiner rejected claim 3 under 35 U.S.C. §112, first paragraph as allegedly "failing to provide an enabling disclosure because the specification does not provide evidence that the claimed biological materials are (1) known and readily available to the public; (2) reproducible from the description *e.g.*, sequenced; or (3) deposited." The Examiner indicates that the rejection may be overcome by depositing the *Salmonella* strains with an International Depository Authority under the provisions of the Budapest Treaty. The Examiner then proceeds, in section 11 of the Office Action (paper 17, page 9) to reject the remainder of claims 1-10 under 35 U.S.C. § 112, first paragraph for lack of enablement. Applicants traverse both rejections and respectfully request reconsideration and withdrawal of the rejections in light of the comments provided below.

The invention, as defined in claim 1, relates to an attenuated Salmonella strain that carries a eukaryotic expression vector for the expression of a heterologous gene (or heterologous gene fragment or an autologous gene or an autologous gene fragment) comprised by the vector within an open reading frame such that the attenuation is suitable for a vaccination of vertebrates.

Thus, the claimed invention is directed to an attenuated Salmonella strain as a **carrier for delivering a eukaryotic expression product** to eukaryotic cells. The Examiner improperly

focuses on the heterologous/autologous gene/fragment to be carried and expressed by the attenuated *Salmonella*, rather than on the vehicle, which is the claimed subject matter. The specification describes how to make and use the claimed vehicles and a failure to recite laundry lists of coding region and diseases does not change that fact, any more than a failure to list suitable wines would result in a failure to enable a claimed corkscrew. By way of further explanation, certain excerpts from the specification are discussed to facilitate a better understanding of the invention.

It is desirable to obtain vaccines that have a low-cost, non-invasive administration and offer prolonged protection against infectious diseases after administration (See specification page 1, lines 4-6). Oral delivery of traditional vaccines often use live attenuated bacterial carriers that express the antigens against which the vaccination is being administered. (see specification page 1, lines 7-8). Using such carriers overcomes various limitations of oral delivery of the antigen alone. (See specification page 1, lines 8-18 for a discussion of some of these advantages). An ***alternative*** to using live attenuated bacterial carriers for vaccination is the use of genetic immunization using DNA vaccines. (See specification page 1, line 19 through page 2 line 6, and the references cited therein). The present invention takes advantage of both the use of an attenuated bacterial carrier and genetic immunization to achieve improved vaccination.

With respect to the rejection of claim 3, Applicants submit that no additional deposit of the Salmonella strains is necessary, as these strains are readily available to those of skill in the art. As a general matter, the present invention is not limited to the particular *Salmonella* strain exemplified in the application. Instead, the combined attenuated bacterial/genetic immunization composition discussed above can be prepared using any *Salmonella* strain that is attenuated using techniques known in the art such that it is rendered useful for vaccinating vertebrates. Indeed, the specification cites several references describing such attenuated strains of Salmonella that were available at the time of filing, and were in use at that time as vaccines, in animals and man. (Page 2, lines 17). As such, contrary to the Examiner's assertions, deposit of a specific *Salmonella* strain is not required for one of skill in the art to make and use the claimed invention. More particularly, the Salmonella strains discussed in the specification are readily available to those of skill in the art. For example, *Salmonella* strains LT2 (ATCC 15277) and

LT2aroA544::Tn10 (ATCC33275), each of which is an attenuated strain of *Salmonella* according to the claims, are readily available from the American Type Culture Collection. Additionally, strain SL 7207 from Dr. Stocker is readily available to those skilled in the art upon request. Because the attenuated strains of *Salmonella* expressly recited in claim 3 were known and available to the public at the time of filing, claim 3 is enabled by the specification.

Applicants further traverse the rejection of claims 1-10 under 35 U.S.C. §112, first paragraph. To satisfy 35 U.S.C. § 112, first paragraph's enablement requirement, the specification must enable a person skilled in the art to make and use **the invention**. The **invention** of claims 1-9 is an attenuated *Salmonella* strain comprising a eukaryotic expression vector for the expression of a heterologous gene or heterologous gene fragment or an autologous gene or autologous gene fragment comprised within an open reading frame. The specification describes that attenuated strains of *Salmonella* suitable for vaccination were known in the art and exemplary strains are described therein. Expression vectors comprising a heterologous gene or heterologous gene fragment or an autologous gene or autologous gene fragment comprised within an open reading frame were known in the art and exemplary vectors are described within the specification. Although known in the art as a routine procedure, introduction of an expression vector into *Salmonella* was described in the specification. A great number of uses for the claimed *Salmonella* strains also are described in the specification. One such use is the introduction of the expression product into a mammalian cell by contacting the cell with a claimed *Salmonella* strain for expression of a heterologous gene or heterologous gene fragment or an autologous gene or autologous gene fragment within the mammalian cell. See pages 8-10 of the specification. Thus, the specification teaches one of ordinary skill in the art how to make and use the **invention** of claims 1-9.

In support of the rejection, the Examiner asserts that the invention's purpose is vaccination and proceeds to focus on the heterologous/autologous gene/fragment rather than the claimed invention. The pending claims are drawn to a vehicle, not to a coding region for an immunogenic polypeptide. Again by way of analogy, a claimed automobile for carrying passengers doe not fail for lack of enablement because the specification fails to specifically identify the passengers. Certainly, the specification teaches one of skill in the art how to make and use the claimed vehicle to express an immunogenic polypeptide. Moreover, coding regions

for peptides whose immunogenicity is unknown but that would be suitable for use in the claimed vehicle could be identified using the type of routine experimentation found acceptable in In re Wands, upon which the Examiner relies. With the enablement inquiry properly focused on the claimed vehicle, it is apparent that each of claims 1-10 is enabled throughout its full scope by the specification as filed.

IV. Rejections under 35 U.S.C. §112, first paragraph should be withdrawn.

In section 12 of the Office Action, claims 1-10 are rejected under 35 U.S.C. §112, second paragraph. Claims 1 and 7 are allegedly unclear for reciting "fragment" of a gene. Again, the invention is directed to a Salmonella strain that acts as a vehicle for transferring an expression vector to a cell. Although even minute fractions of a gene, as suggested by the Examiner, would be less desirable to use than larger fragments, even such minute fragments are included in the present claims provided they are "within an open reading frame." Thus, claim 7 properly avoids a size or length limitation relating to the heterologous/autologous gene/fragment.

Claim 6 has been amended to delete recitation of "facultatively" as suggested by the Examiner and spell out the identity of "CMV", which is well known to those of skill in the art.

Regarding the use of "variant" in claim 9, the term is known in the art to refer to a variation of a wild-type gene sequence. Such variations include, *inter alia,* truncated variants and mutated variants. The variants of claim 9 are truncations of the recited genes.

V. Conclusion

For the foregoing reasons, each of presently pending claims 1-10 is believed to be in condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejections of the claims and to pass this application to issue.

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Respectfully submitted,

By _____

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APPENDIX A

Version With Markings to Show Changes Made

In the specification:

In the paragraph starting at line 28 of page 2:

Further, the *Salmonella* strain according to the invention can be a *S. typhi* [*typhii*] strain, especially *S. typhi* [*typhii*] Ty21a.

In the paragraph starting at line 31 of page 2:

According to the invention *Salmonella* strains are comprised, wherein the eucaryotic expression vector is or can be derived from the known plasmid pCMVβ which comprises
-the structural gene of β-galactosidase (β-gal)
-under control of the human cytomegalovirus (CMV) immediate early promoter comprised by the plasmid pCMVβ per se and includes
-a splice donor,
-two splice acceptor sites in between the promoter and the β-galactosidase gene, and facultatively
-the polyadenylation site of SV40.--

In the paragraph starting at line 12 of page 3:

According to the invention *Samonella* strains are comprised wherein the heterologous gene is selected from the group consisting of
- the *Esherichia coli*-β-galactosidase gene (LacZ-gene),
- a non-hemolytic truncated variant of the *Listeria monocytogenes*-listeriolysine [listerio lysine] gene (hly gene)
and
- a truncated variant of the *Listeria monocytogenes*-actA gene (actA gene).

On page 15, the description of Fig. 1:

Fig. 1. Induction of cytotoxic T cells in mice orally immunized with 10^8 *S. typhimurium aroA* carrying eukaryotic expression plasmids which encode listeriolysin or ActA. Mice were immunized either four times with two week [intervals] intervals (A, B, D, E) or once(C[;], F) with *Salmonella* carrying pCMVhly (A-C) or pCMVActA (D-F) and spleen cells were restimulated once *in vitro* with a synthetic peptide comprising AA91-99 of listeriolysin (A-C) or with a mixture of purified ActA and hemolytically active listeriolysin which results in the class I presentation of ActA due to the pore-forming activity of listeriolysin (Darji et al., 1995a; Darji et al., 1997). Restimulated T cells were tested with radiolabelled P815 target cells at an effector to target ratio of 10:1. **Fig. 1A:** Specificity of the anti-listeriolysin cytotoxic response. Target cells were sensitized with henegglysozyme (HEL), peptide AA 91-99 of listeriolysin (pLLO) or control peptide of nucleoprotein of influenza virus (pNP). Displayed is the experiment with spleen cells from week 5 shown in panel B. Similar specificity was observed at all other time points. **Fig. 1B.** Kinetic of the cytotoxic response of mice immunized four times with pCMVhly. The arrows indicate the booster immunizations. **Fig. 1C.** Kinetic of the cytotoxic response of mice immunized once with pCMVhly. **Fig. 1D.** Specificity of the anti-ActA cytotoxic response. Target cells were sensitized with a mixture of Act A and listeriolysin (ActA + LLO), HEL, and listeriolysin (HEL + LLO) or listeriolysin alone (LLO). Displayed is the experiment with restimulated spleen cells from week 5 shown in panel E. Similar specificity was observed at other time points and including other synthetic peptides of various sources. **Fig. 1E.** Kinetic of the cytotoxic T cell response in mice immunized four times pCMVActA. Arrows indicate booster immunizations. **Fig. 1F.** Kinetic of the cytotoxic T cell response in mice immunized once with pCMVActA. The specificity of the cytotoxic response was further assessed by testing the spleen cells of mice immunized in a similar way with pCMVß (ß-gal) on target cells sensitized with pLLO, ActA plus listeriolysin or a ß-gal expressing transfectant of P815 (data not shown). Similarly, a specific cytotoxic T cell response was observed against ß-gal[.], but the kinetic was not followed as systematically as for the two other antigens.

On pages 15-16, the description of Fig. 2:

Fig. 2. Induction of cytotoxic T cells in mice orally immunized with 10^8 *S. typhimurium aroA* carrying eukaryotic expression plasmids which encode listeriolysin or ActA. Spleen (SPC) and

lymph node cells (LNC) from the same mice tested for cytotoxic T cell responses displayed in Fig. 1 were tested for T helper responses. Mice were immunized either four times (A, B, C, E) or once (C[;], F) with *Salmonella* carrying pCMVhly (A-C) or pCMVActA (D, F) and restimulated *in vitro*. After two days proliferation was tested by incorporation of ^{3}H-Thymidine. **Fig. 2A:** Specificity of the proliferative response of spleen cells from mice immunized with pCMVhly. T cells tested were the same as those displayed in panel B at week 11. Similar results were obtained at other time points. **Fig. 2B:** Kinetics of the proliferative response of spleen and lymph node cells from mice immunized four times with pCMVhly. Arrows indicate the booster immunizations. **Fig. 2C:** Kinetics of proliferative response of spleen and lymph node cells from mice immunized once with pCMVhly. **Fig. 2D:** Specificity of proliferative response of spleen cells from mice immunized four times with pCMAVActA. T cells tested were the same as those displayed in panel D at week 11. Similar results were obtained at other time points. **Fig. 2E:** Kinetics of the proliferative response of spleen and lymph node cells immunized four time with pCMVActA. Arrows indicate booster immunizations. **Fig. 2F:** Kinetics of the proliferative response of spleen and lymph node cells from mice immunized once with pCMVActA. Similarly, spleen and lymph node cells from mice immunized with pCMV ß (ß-gal) never reacted with either listeriolysin or ActA but could respond to [restimmulation] restimulation with ß-gal (data not shown).

On page 16, the description of Fig. 3:

Fig. 3. Kinetics and subclass distribution of specific serum IgG from mice orally immunized with *S. typhimurium aroA* carrying eukaryotic expression plasmids which encode listeriolysin, ActA or ß-gal. Sera from the same mice tested for cytotoxic and proliferative T cell responses displayed in Figs. 1 and 2 were used and assayed in specific ELISA's. Mice were immunized four time (**Fig. 3A**[A]) or once (**Fig. 3B**[B]) with pCMVhly, pCMVActA or pCMVß, respectively, and pooled sera were tested for antigen specific serum IgG. To assess specificity, all sera were tested on all three antigens. Reactivity was only observed against the immunizing antigen (data not shown). Identical results were obtained by immunoblotting using the same antigens (data not shown). The subclass distribution 11 weeks after the first immunization was

determined from the sera of individual mice immunized four time (closed symbols) or once (open symbols) with either pCMVhly (**Fig. 3C**[C]) or pCMVActA (**Fig. 3D**[D]).

Starting on pages 16, the description of Fig. 4:

Fig. 4. Oral immunization with *S. typhimurium aroA* carrying the eukaryotic express plasmid which encodes listeriolysin induces a protective immune response, whereas immunization with bacteria carrying the expression plasmid for ActA is not protective. Groups of six mice were immunized four times with two week intervals (**Fig. 4A**[A]) or only once (**Fig. 4B**[B]) with *Salmonella* carrying pCMVhly, pCMVActA or pCMVß and challenged with a lethal dose of 5 x 10^4 *L. monocytogenes* EGD (10 x LD_{50}) intravenously. Mice that had been immunized only once with pCMVhly became moribund after two days. However, four of them recovered and survived in good condition until the experiment was terminated two weeks later.

On page 17, the description of Fig. 5:

Fig. 5 Comparison of orally induced immune response elicited with *Salmonella* harboring prokaryotic or eukaryotic expression plasmids for ß-gal. Mice were immunized with *Salmonella* harboring either the eukaryotic expression plasmid pCMVß or the plasmid pAH97 that constitutively expressed ß-gal from the Pr and Ps promotor of *XylS* of *Pseudomonas putida*. Bacteria harboring the eukaryotic vector were administered orally once (●), whereas bacteria express ß-gal under the control of the prokaryotic promoter were administered either once (♦) or four times with two week intervals (▼). The arrows indicate the time of booster immunizations. **Fig. 5A:** Cytotoxic response or restimulated spleen cells tested at an effector to target ratio of 10:1. The ß-gal expressing transfectant P13.1 was used as target in the JAM assay. **Fig. 5B.** Proliferative helper T cell response of spleen cells with isolated [b-gal] ß-gal as antigen. **Fig. 5C:** Antibody response against ß-gal from pooled sera measured by ELISA. Data displayed in **Fig. 5A-C** [A-C] were obtained with cells or sera from the same mice. All assays were performed as described in figures 1-3.

On page 22, the paragraph under the heading "ELISA":

To evaluate the levels of immunoglobulins against LLO, Act-A and ß-gal serum specimens, 96-well ELISA plates (Maxisorp, Nunc) were coated with 0.5 µg/ml purified protein overnight at 4° C. Plates were washed three times with PBS/0.05% polyoxyethylene non-ionic detergent TWEEN [Tween] 20 and then blocked with 3% BSA-PBS for 2 h at 37°C. Following two washes with PBS/0.05% polyoxyethylene non-ionic detergent TWEEN [Tween] 20, serum samples if a 1:100 dilution were added to individual wells and incubated for 2-3 h at 37°C. Plates were washed above and biotinylated goat anti-mouse Ig (Dianova, Hamburg, Germany) in 1% BSA-PBS was added to each well and incubated for 1 h at 37°C. After three washes with PBS/0.05% polyoxyethylene non-ionic detergent TWEEN [Tween] 20, horseradish peroxidase conjugated streptavidin (Dianova, Hamburg, Germany) in 1% BSA-PBS was added to each well and incubated for 1 h at 37°C. Plates were washed as above, developed with *o*-Phenylene diamine as substrate and measured in an ELISA reader at 490nm. For antigen specific IgG subclasses determination, peroxidase conjugated goat anti mouse IgG, IgG2A, IgG2b and IgG3 (Caltag laboratories, CA, USA) were used.

On page 23, the paragraph under the heading "RNA isolation and RT-PCR":

In order to test for expression of ß-gal transferred into the eukaryotic host cells via *Salmonella,* the mRNA was probed for the presence of splice products derived from the splice donor and acceptors of the expression plasmid. To this end, PECs were infected *in vitro* at an MOI of 10 with *S. typhimurium aroA* harboring the eukaryotic expression vector pCMBß and RNA was extracted as described (Chomczynski and Sacchi, 1987). RT-PCR of isolated RNA was performed. Briefly, 10 µg of isolated total cellular RNA was resuspended in 20 µl of DEPC-H_2O and incubated for 5 min at 70° C with 10 µl of buffer containing 6 µl of reverse transcriptase buffer (250 mM Tris-HCl, 375 mM KCl, 15mM $MgCL_2$); 0,4mM dNTPs; 0,05 U random hexamers (Pharmacia, Uppsala, Sweden); and 1 mM DTT. Samples were spun down for 2 min at 15,000 rpm and 40 U RNAsin ribonuclease inhibitor (Promega) together with 200 U Superscript reverse transcriptase (Gibco, BRL) were added. RNA was reverse transcribed for 445 min at 37°C and the reaction was stopped by heating the samples at 95°C for 1 min followed by a short incubation on ice. Subsequently, 500 ng of cDNA product was amplified by PCR in a

final volume of 50 µl containing 0,2mM dNTP, 20mM DTT, 3 µM of each of the 5' and 3' primer, 5 µl of 10x PCR buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 1% gelatine, 1,5mM $MgCL_2$, 1% polyoxyethylene non-ionic detergent TRITON [Triton] X-100) and 5 U AmpliTaq-DNA-polymerase (Perkin Elmer). PCR was performed with an initial denaturation step of 10 min at 85°C followed by 35 cycles of 20 sec denaturation at 95°C, 30 sec annealing at 60°C and 30 sec extension at 72°C. The amplification products were visualized under a UV lamp after electrophoresis of a 15 µl aliquot of the reaction mixture on a 2% (w/v) agarose gel containing 0,5 µg/ml of ethidium bromide. The primer pair was designed in such a way that the presence of splice products should be indicated by a 190 bp and/or a 125 bp fragment. The identity of the resumable splice product was confirmed by sequencing the fragments after isolation on a preparative agarose gel. The primer paid used for amplification and sequencing - SV40 forward: 5'-GGATCCGGTACTCGAGGAAC-3' (SEQ ID NO:5), SV40 reverse: 5'-GCTTTAGCAGGCTCTTTCG-3' (SEQ ID NO:6).

In the claims:

1. [Amended] An attenuated *Salmonella* strain comprising a eukaryotic [eucaryotic] expression vector for the expression of a heterologous gene or heterologous gene fragment or an autologous gene or autologous gene fragment comprised by the vector within an open reading frame wherein the attenuation is suitable for a vaccination of vertebrates.

4. [Amended] The *Salmonella* strain according to claim 1 wherein the strain is a *S. typhi* [*typhii*] strain.

5. [Amended] The *Salmonella* strain according to claim 4 wherein the strain is *S. typhi* [*typhii*] Ty21a.

6. [Amended] The Salmonella strain according to claim 1 wherein the eukaryotic [eucaryotic] expression vector is derived from plasmid pCMVß, wherein the plasmid comprises:

a) a structural gene of ß- galactosidase (ß- gal) under the control of a human cytomegalovirus (CMV) immediate early promoter,

b) a splice donor,

c) two splice acceptor sites between the promoter and the ß-galactosidase gene, and [facultatively]

d) a polyadenylation site of SV40.